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## 3-Phenylcoumarin derivatives selectively modulate different steps of reactive oxygen species production by immune complex-stimulated human neutrophils

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## ABSTRACT

Immune complex (IC) deposition in tissues triggers the release of harmful oxidant and lytic compounds by neutrophils. We examined how ten 3-phenylcoumarin derivatives affect the reactive oxygen species (ROS) production by IC-stimulated human neutrophils. Most of the 3-phenylcoumarins inhibited the luminol-enhanced chemiluminescence (CL-lum) more strongly than they inhibited the lucigenin-enhanced chemiluminescence (CL-luc), without clear signs of toxicity. The most effective CL-lum inhibitors, 6,7-dihydroxy-3-[3',4'-methylenedioxyphenyl]-coumarin (**5**) and 6,7-dihydroxy-3-[3',4'-dihydroxyphenyl]-coumarin (**19**), also inhibited myeloperoxidase activity more potently and had higher hypochlorous acid scavenging ability, but did not affect the NADPH-oxidase activity. The type, number, and position of the substituent influenced the pharmacological effects of 3-phenylcoumarins; however, the structural requirements for CL-lum and CL-luc inhibition were a little different. Compounds **5** and **19** are promising prototypes of therapeutic molecules to modulate ROS production by neutrophils in IC-mediated inflammatory diseases.

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## 1. Introduction

The organism continuously produces antigen–antibody immune complexes (IC) in response to foreign antigens, infection, and tissue injury. By binding to erythrocytes, IC are transported to the spleen and liver and further cleared from the circulation. However, increased IC formation and defects in the IC clearance mechanism culminate in IC deposition in vessels and tissues, which activates the complement system and chemotaxis of inflammatory cells [1,2]. Fcγ and complement receptors mediate the neutrophil activation at the inflammatory site,

triggering phagocytosis, degranulation, and reactive oxygen species generation (ROS) via the NADPH oxidase complex and myeloperoxidase (MPO) [3]. Microbial killing depends on inflammatory cells releasing oxidant and lytic compounds; however, these compounds can damage the surrounding tissues and impair the target organ function [4]. For instance, IC deposition in tissues is strongly associated with rejection of transplanted organs and initial response of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and rheumatic fever [2].

Neutrophils play a fundamental role in host defense and acute inflammatory responses. To regulate autoimmune inflammation, it is important that neutrophils produce adequate amounts of ROS. Moreover, neutrophils have an important part in chronic inflammatory responses [5–7]. In this sense, modulating neutrophil ROS production is a promising therapeutic strategy to manage inflammation and reduce the deleterious effects of the over-activation of immune system cells in many diseases [5,8,9]. Our research team has investigated the pharmacological effects of natural and synthetic compounds like flavonoids [10–12], coumarins [13–15], and sesquiterpene lactones [16]. We have sought to understand structure–activity relationships, unravel the mechanisms of action of these compounds, and discover prototypes of compounds that can act as modulators of the effector functions of neutrophils in IC-mediated inflammatory diseases.

**Abbreviations:** AUC, area under the curve; CL, chemiluminescence; CL-luc, lucigenin-enhanced chemiluminescence; CL-lum, luminol-enhanced chemiluminescence; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; HBSS, Hank's balanced saline solution; HBSS-gel, Hank's balanced saline solution supplemented with 0.1% gelatin; IC, immune complex; IC<sub>50</sub>, concentration inhibiting a biological response by 50%; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; *p*-AB, *p*-aminobenzohydrazide; ROS, reactive oxygen species; TMB, 3,3',5,5'-tetramethylbenzidine.

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Coumarins constitute an important class of secondary metabolites that occur in plants and microorganisms, and they display a wide range of biological activities [17,18]. The last ten years have seen intense work on the chemical modification of the coumarin skeleton, in order to obtain more potent antioxidant and anti-inflammatory compounds [13,15,19–23]. The addition of a 3-phenyl ring and hydroxyl groups to the coumarin molecule yields hydroxylated 3-phenylcoumarin derivatives, known for their increased antioxidant effect [14,24] and ability to inhibit peroxidases [14] and lipoxygenase [24]. These properties reveal the potential anti-inflammatory activity of these derivatives.

Higher plants produce 3-phenylcoumarins by the same biosynthetic pathway that flavonoids are generated, so these coumarins are considered a subclass of isoflavonoids [25]. 3-Phenylcoumarins exhibit antimicrobial [26,27], antiviral [28], antidepressant [29], anticoagulant [30], and vasorelaxant [31] actions. Furthermore, they inhibit the glyceraldehyde 3-phosphate dehydrogenase of *Trypanosoma cruzi* [32], monoamine oxidase [33] and tyrosinase [34].

Recently, we screened the inhibitory effect of a series of 3-phenylcoumarin derivatives in the oxidative metabolism of rabbit IC-stimulated neutrophils [15]. The qualitative and quantitative structure–activity relationship analysis allowed us to select promising modulators of this neutrophil effector function. To continue investigating the immunomodulating potential of 3-phenylcoumarins, in this study we evaluated the inhibitory effect of ten selected 3-phenylcoumarin derivatives bearing free and acetylated hydroxyl groups on  $O_2^{\bullet-}$  generation and total ROS production by IC-stimulated human neutrophils. We also investigated possible mechanisms underlying the pharmacological effect of these compounds, such as their inhibitory activity toward NADPH oxidase and MPO as well as their hypochlorous acid (HOCl) scavenging and cytotoxic potential.

## 2. Results

### 2.1. Inhibition of ROS production by neutrophils

First, we used the CL-luc and CL-lum assays to screen the inhibitory effect of ten 3-phenylcoumarin derivatives (at 20  $\mu$ M) on the  $O_2^{\bullet-}$  and

total ROS generation by IC-stimulated neutrophils, respectively. Compared with the control, all the tested compounds had a significant inhibitory effect on both CL-lum and CL-luc (Fig. 2a and b). In general, the 3-phenylcoumarin derivatives inhibited the neutrophil CL-lum more strongly than they inhibited the neutrophil CL-luc; only compounds **1**, **7**, and **9** inhibited CL-lum and CL-luc to the same degree.

Next, we selected the compounds that inhibited CL-lum (**1**, **5**, **6**, **19**, and **20**) or CL-luc (**1**, **5**, **19**, and **20**) by more than 50% and investigated their mechanism of action. The aforementioned derivatives inhibited the IC-stimulated neutrophil CL-lum (Fig. 2c) and CL-luc (Fig. 2d) in a concentration-dependent manner; their  $IC_{50}$  values are reported in Table 2.

Compounds bearing the catechol group (**5** and **19**) inhibited CL-lum the most effectively, but acetylation of their free hydroxyls decreased their inhibitory potency by three- to fourfold (**5** vs. **6**; **19** vs. **20**) (Table 2). Compounds **5** and **19** had significantly lower  $IC_{50}$  values than quercetin. The non-substituted compounds **7** and **8** and the monosubstituted compounds **9** and **10** were the least active — they inhibited nearly 30% of CL-lum. Compounds **1** and **2** inhibited around 70% and 45%, respectively, of CL-lum at the highest concentration tested herein (20  $\mu$ M) (Fig. 2a).

Compound **5** inhibited CL-luc the most strongly: it was three times more effective than quercetin and five to six times more effective than compounds **1**, **19**, and **20**, which had similar inhibitory effects (Table 2). The hydroxylated 3-phenylcoumarin derivatives had significantly higher inhibitory activity than their acetylated counterparts (**1** vs. **2**; **5** vs. **6**; **9** vs. **10**) (Fig. 2b). Compounds **8** and **10** were the least effective. Compounds **2**, **6**, **7**, and **9** inhibited only 30–40% of CL-luc at 20  $\mu$ M (Fig. 2b).

For most of the 3-phenylcoumarin derivatives, the presence of the 3',4'-methylenedioxy group enhanced the inhibition of neutrophil CL-luc (**1** vs. **9**; **2** vs. **10**; **5** vs. **19**; **7** vs. **8**). In the case of the monosubstituted 3-phenylcoumarin derivatives, this same substituent increased the inhibition of neutrophil CL-lum (**1** vs. **9**; **2** vs. **10**) (Fig. 2, Table 2).

Together, these results suggest that the type, number, and position of the substituent influence the inhibitory effect of 3-phenylcoumarins on ROS production by IC-stimulated neutrophils. However, the structural

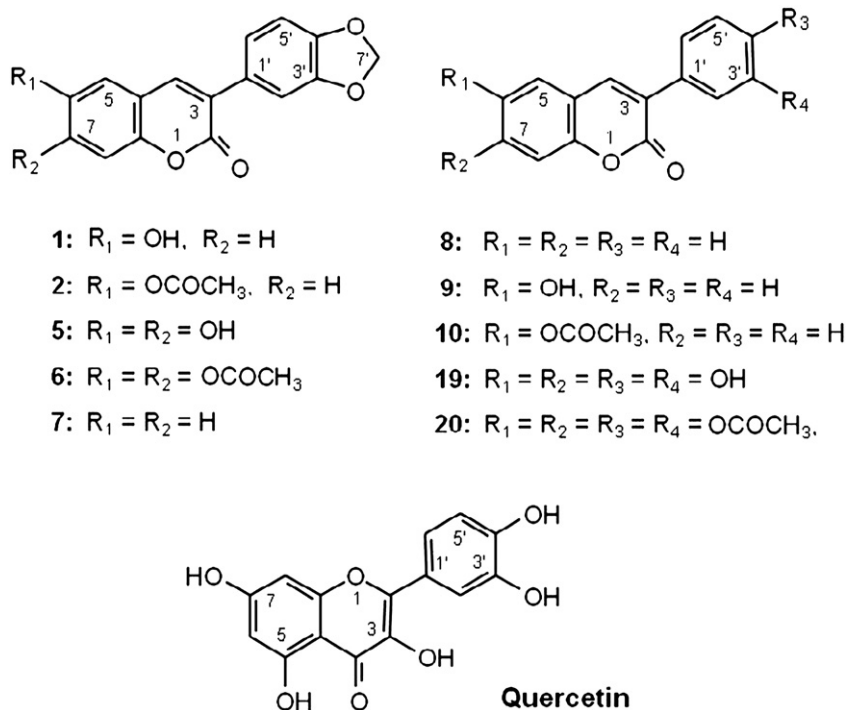
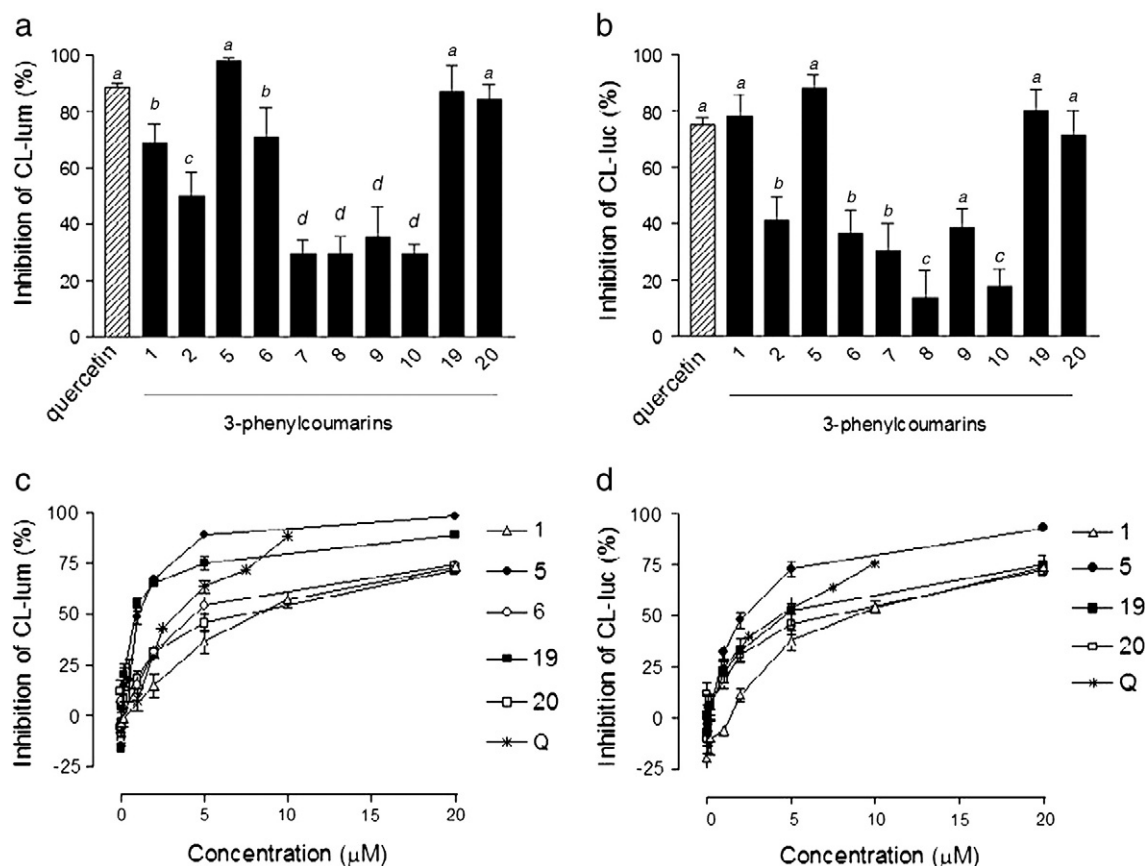


Fig. 1. Chemical structures of 3-phenylcoumarin derivatives and quercetin.



**Fig. 2.** Inhibitory effect of 3-phenylcoumarin derivatives on the human neutrophil reactive oxygen species generation, triggered by immune complexes, and assessed by luminol- and lucigenin-enhanced chemiluminescence assays (CL-lum and CL-luc, respectively). Graphs a–b: Comparative effect of the ten 3-phenylcoumarins at 20 µM and quercetin at 10 µM on neutrophil CL-lum (graph a) and CL-luc (graph b). Graphs c–d: Concentration-dependent plots of quercetin (Q) and the 3-phenylcoumarins showing the highest inhibitory effects on neutrophil CL-lum (graph c) and CL-luc (graph d). Results are expressed as mean  $\pm$  SD of at least three independent experiments, assayed in duplicate. Statistics: values not sharing the same letter (a–d) are significantly different from each other ( $p < 0.05$ ; ANOVA and Tukey's *post-hoc* test).

requirements for CL-lum and CL-luc inhibition among the 3-phenylcoumarin derivatives were a little different.

## 2.2. Cytotoxicity studies

Compared with the control, the ten 3-phenylcoumarin derivatives and quercetin did not induce significant LDH release or decrease neutrophil viability (Table 1). Thus, these compounds are not cytotoxic under the assessed conditions.

## 2.3. Inhibition of NADPH oxidase activity

We measured the cellular  $O_2$  consumption, in order to find out whether the 3-phenylcoumarin derivatives modulate the NADPH oxidase activity in IC-stimulated neutrophils. We tested compounds 1, 5, 19, and 20, which displayed the highest inhibitory effects on the IC-stimulated neutrophil CL-luc. The four compounds inhibited the NADPH oxidase activity in a similar way, but they were significantly less efficient than quercetin and DPI ( $p < 0.05$ , Table 3). Therefore, the tested 3-phenylcoumarin derivatives have low inhibitory effect on the NADPH oxidase activity of IC-stimulated neutrophils.

## 2.4. Inhibition of myeloperoxidase activity

We investigated whether the 3-phenylcoumarin derivatives with the highest inhibitory effects on the IC-stimulated neutrophil CL-lum (1, 5, 6, 19, and 20) inhibited MPO activity.

Surprisingly, compound 19 inhibited MPO activity twice more effectively than the other tested compounds ( $p < 0.05$ ), including the

standard MPO inhibitor *p*-AB and the flavonoid quercetin (Table 3). Concentration-dependent plots of compound 19 and *p*-AB revealed similar inhibitory effects at concentrations up to 12 µM, followed by increased effect of compound 19 at higher concentrations (data not shown).

**Table 1**

Viability of neutrophils after treatment with 3-phenylcoumarin derivatives and quercetin<sup>a</sup>.

Compound <sup>b</sup>	Viable cells (%) <sup>c</sup>	Released LDH (%) <sup>d</sup>
HBSS	97.5 $\pm$ 0.9	5.7 $\pm$ 1.1
DMSO	96.6 $\pm$ 1.3	6.7 $\pm$ 2.4
1	95.5 $\pm$ 2.0	3.4 $\pm$ 1.2
2	96.5 $\pm$ 1.0	3.4 $\pm$ 1.5
5	95.0 $\pm$ 2.0	6.7 $\pm$ 2.6
6	95.3 $\pm$ 2.9	5.1 $\pm$ 0.7
7	96.0 $\pm$ 1.0	6.5 $\pm$ 2.8
8	96.7 $\pm$ 1.0	6.5 $\pm$ 2.7
9	97.9 $\pm$ 0.7	3.9 $\pm$ 1.3
10	96.4 $\pm$ 1.3	2.9 $\pm$ 0.5
19	95.0 $\pm$ 1.8	8.4 $\pm$ 1.0
20	95.0 $\pm$ 2.5	5.9 $\pm$ 2.2
Quercetin	96.6 $\pm$ 3.2	3.7 $\pm$ 1.5

<sup>a</sup> Data represent the mean  $\pm$  SD of three independent experiments assayed in duplicate.

<sup>b</sup> HBSS: Hank's Balanced Salt Solution (untreated cells); DMSO: dimethyl sulfoxide (0.1% v/v; vehicle control); 3-phenylcoumarin derivatives 1–20 tested at 20 µM; quercetin tested at 10 µM.

<sup>c</sup> Cell viability determined by the Trypan Blue exclusion test with a total of 200 cells counted for each sample.

<sup>d</sup> Values represent relative amounts of LDH released into the supernatant compared with neutrophils completely lysed by Triton X-100.

**Table 2**

Inhibitory effect of the 3-phenylcoumarin derivatives on the reactive oxygen species generation by immune complex-stimulated human neutrophils.

Compound	IC <sub>50</sub> (μM)	
	CL-lum	CL-luc
<b>1</b>	10.44 ± 3.14 <sup>a</sup>	7.48 ± 2.45 <sup>a</sup>
<b>5</b>	1.05 ± 0.16 <sup>b,d</sup>	1.28 ± 0.05 <sup>b,*</sup>
<b>6</b>	4.06 ± 0.72 <sup>c</sup>	> 20
<b>19</b>	0.73 ± 0.23 <sup>b</sup>	6.98 ± 2.61 <sup>a,*</sup>
<b>20</b>	2.01 ± 0.85 <sup>d,e</sup>	8.32 ± 1.51 <sup>a,*</sup>
Quercetin	3.37 ± 0.43 <sup>c,e</sup>	4.05 ± 0.47 <sup>c</sup>

IC<sub>50</sub> is the concentration inhibiting the luminol- or lucigenin-enhanced chemiluminescence (CL-lum or CL-luc, respectively) by 50%. Data are expressed as mean ± SD of at least three independent experiments, assayed in duplicate. *Statistics*: values in a column not sharing the same letter (a–e) are significantly different from each other (p < 0.05; ANOVA and Tukey's post-hoc test).

\* p < 0.01 (CL-lum vs. CL-luc; Student's t test).

Compound **5** inhibited MPO activity similarly to *p*-AB but more effectively than quercetin (p < 0.05). Compound **1** was as effective as quercetin. Compared with the control, the acetylated 3-phenylcoumarin derivatives **6** and **20** did not inhibit MPO significantly under the assessed conditions. Together, these results suggest that the hydroxylation pattern of the phenolic compound is important for efficient MPO activity inhibition.

### 2.5. Hypochlorous acid scavenging assay

We assayed the HOCl scavenging potential of the five 3-phenylcoumarin derivatives (compounds **1**, **5**, **6**, **19**, and **20**) with the highest inhibitory effects on the neutrophil CL-lum in a cell-free system. The tested derivatives showed concentration-dependent effects, and compound **19** scavenged HOCl the most effectively (Table 3). Compound **5** scavenged HOCl as effectively as quercetin, significantly more effectively than compounds **1** and **20**, but around three times less effectively than compound **19**. Although we were not able to determine IC<sub>50</sub> for compound **6**, concentration-dependent plots revealed that it scavenged around 45% of the HOCl added to the reaction medium at 12 μM, the highest concentration tested (data not shown). Together, these results suggest that the number and position of free hydroxyls determine the biological activity of 3-phenyl coumarins, and that the acetylated derivatives also scavenge HOCl effectively.

## 3. Discussion

In the present study we assessed whether ten 3-phenylcoumarin derivatives inhibit the ROS generation by IC-stimulated human neutrophils,

**Table 3**

Inhibitory effect of the 3-phenylcoumarin derivatives on the activities of NADPH oxidase and myeloperoxidase and their HOCl scavenging potential<sup>a</sup>.

Compound <sup>b</sup>	NADPH oxidase inhibition (%)	MPO inhibition (%)	HOCl scavenging IC <sub>50</sub> (μM) <sup>c</sup>
<b>1</b>	6.17 ± 2.84 <sup>a</sup>	18.76 ± 5.37 <sup>a</sup>	5.01 ± 0.83 <sup>a</sup>
<b>5</b>	3.20 ± 1.38 <sup>a</sup>	34.25 ± 5.43 <sup>b</sup>	3.75 ± 0.80 <sup>b</sup>
<b>6</b>	n.t.	1.66 ± 0.92 <sup>c</sup>	> 12
<b>19</b>	4.31 ± 2.08 <sup>a</sup>	53.92 ± 4.09 <sup>d</sup>	1.03 ± 0.23 <sup>c</sup>
<b>20</b>	4.68 ± 2.38 <sup>a</sup>	6.44 ± 2.62 <sup>e</sup>	5.02 ± 0.65 <sup>a</sup>
Quercetin	18.25 ± 4.93 <sup>b</sup>	23.31 ± 3.99 <sup>a</sup>	3.19 ± 0.08 <sup>b</sup>
DPI	99.90 ± 0.10 <sup>c</sup>	n.t.	n.t.
<i>p</i> -AB	n.t.	31.20 ± 3.15 <sup>b</sup>	n.t.

<sup>a</sup> Data expressed as mean ± SD of three independent experiments assayed in duplicate; n.t.: not tested.

<sup>b</sup> Quercetin and 3-phenylcoumarins tested at 20 μM; DPI: diphenyliodonium chloride (1 μM, specific inhibitor of NADPH oxidase), *p*-AB: *p*-aminobenzohydrazide (20 μM, specific inhibitor of MPO).

<sup>c</sup> IC<sub>50</sub> is the concentration that scavenged 50% of the HOCl added to the reaction medium. *Statistics*: values in a column not sharing the same letter (a–e) are significantly different from each other (p < 0.05; ANOVA and Tukey's post-hoc test).

investigated some of the underlying mechanisms of action of these compounds, and discuss the most relevant structural features for each assessed biological activity. We used the chemiluminogenic probes lucigenin and luminol to study how the 3-phenylcoumarin derivatives affect the different steps of ROS generation by neutrophils. Lucigenin specifically detects O<sub>2</sub>•<sup>−</sup>, the first ROS produced via the NADPH oxidase complex; luminol measures the overall ROS generation, with higher sensitivity for the ROS originated from the MPO–H<sub>2</sub>O<sub>2</sub>–halide system in subsequent steps [35].

All the tested compounds inhibit neutrophil CL-luc and CL-lum to some extent, but this effect is not related to toxicity of the 3-phenylcoumarins on these cells, at least under the assessed conditions. This result agrees with a previous work that reported the lack of 3-phenylcoumarins cytotoxicity on rabbit neutrophils [15].

In general, the presence of free hydroxyls determines CL-luc and CL-lum inhibition. Indeed, acetylation of this group decreases the inhibitory potency of most derivatives. In addition, the most active compounds (**5**, **6**, **19**, and **20**) bear at least one catecholic or *o*-diacetoxy group, indicating that this spatial disposition of the substituents favors the inhibitory effect. The catechol group improves the inhibitory effect of coumarins on superoxide anion generation by rat neutrophils [17] as well as the antioxidant activity of coumarins [36]. On the other hand, addition of the acetyl group to monohydroxylated compounds does not affect inhibition of the rabbit neutrophil respiratory burst [13], but acetylation of the free hydroxyls of polyphenols increases the anti-inflammatory effect of these molecules. This structural modification enhances inhibition of prostaglandin synthesis and nitric oxide production by macrophages and prevents ICAM-1 expression by endothelial cells [37,38].

Interestingly, most compounds bearing the 3',4'-methylenedioxy group (**1**, **2**, **5**, and **7**) inhibit CL-luc significantly more effectively than the ones lacking this group (**8**, **9**, **10**, and **19**). Substitution of the 3',4'-diacetoxy group (**20**) for the 3',4'-methylenedioxy group (**6**) suppresses the inhibitory effect of the 3-phenylcoumarin derivatives on CL-luc. These results suggest that the presence of the catechol group as well as the size and lipophilicity of the molecule are important for CL-luc inhibition. It is noteworthy that (1) compounds **7** and **8** do not have any substituents; (2) compounds **2** and **10** contain one or two hydroxyls; and (3) compounds **1**, **5**, **9**, and **19** bear one or two hydroxyls as well as a 3',4'-methylenedioxy group, which makes the molecule more lipophilic. Moreover, the 3',4'-methylenedioxy group attached to the 3-phenyl ring forms a planar structure, the 3-piperonyl group, which can favor interaction of the molecule with the membrane lipid bilayer, thus facilitating entrance of the 3-phenylcoumarin derivative into the intracellular environment.

Regarding CL-lum, the additional presence of the 3',4'-methylenedioxy group improves the inhibitory ability of the mono-substituted compounds (**1** vs. **9**, **2** vs. **10**), but it does not influence the inhibitory ability of the non-substituted (**7** vs. **8**) or the 6,7-dihydroxylated (**5** vs. **19**) compounds. Moreover, substitution of the 3',4'-diacetoxy group (**20**) for the 3',4'-methylenedioxy group (**6**) slightly decreases the inhibitory effect on CL-lum. These data indicate that the presence of vicinal hydroxyl groups, free or acetylated, is the most important structural feature for significant CL-lum inhibition.

Compound **5**, which bears 6,7-dihydroxyl and 3',4'-methylenedioxy groups, inhibits the two steps of neutrophil ROS generation the most effectively; it also gives similar IC<sub>50</sub> values for CL-lum and CL-luc. Therefore, the hydrogen-donor ability and lipophilicity of the molecule contribute to inhibiting the two main steps of ROS production by IC-stimulated human neutrophils. Compounds **6**, **19**, and **20** seem to modulate this neutrophil function more selectively, since their CL-lum IC<sub>50</sub> values are more than fourfold lower than their CL-luc IC<sub>50</sub> values.

Increased lipophilicity improves the inhibitory effect of flavonoids on the oxidative metabolism of IC- and phorbol myristate acetate-stimulated neutrophils [12,39]. However, correlating the physicochemical property and biological activity is no easy task. The inhibition of rabbit neutrophil



CL-luc and the lipophilicity of 3-phenylcoumarins correlate following an inverted parabola [15]. In other words, upon increasing lipophilicity the biological activity first decreases to a minimum and then rises.

Increased lipophilicity of catechin derivatives [39] and acetylated quercetin derivatives [37] augments their bioavailability and favors their interaction with intracellular targets. Esterification of fluorescent probes such as Fluo-3 and Fura-2 enhances the lipophilicity and intracellular bioavailability of these derivatives. The ester form of the probe readily crosses the plasma membrane, and intracellular esterases cleave the ester bond. This culminates in hydrophilic products that accumulate in the cytoplasm [40]. Considering the mechanism described for fluorescent probes, we believe that esterases partially hydrolyze the acetylated 3-phenylcoumarins, resulting in intracellular products with different biological actions from those of the totally hydroxylated or acetylated compounds.

Comparing the inhibitory effect of the 3-phenylcoumarin derivatives on neutrophil CL-lum and CL-luc, we observe that most compounds (**2**, **5**, **6**, **8**, **10**, **19**, and **20**) inhibit CL-lum more strongly. This suggests that the aforementioned derivatives do not significantly act at the beginning of neutrophil ROS production, but they probably affect the latter steps of this process. To test this hypothesis, we evaluated the ability of selected 3-phenylcoumarins to inhibit the neutrophil NADPH oxidase and MPO activities, as well as their HOCl scavenging potential.

NADPH oxidase is an important therapeutic target in inflammatory diseases involving neutrophils [9,41,42]. The 3-phenylcoumarin derivatives **1**, **5**, **19**, and **20**, the strongest inhibitors of CL-luc, only slightly affect (5–7%) O<sub>2</sub> consumption by IC-stimulated neutrophils. Therefore, these compounds virtually do not act on the assembly or catalytic activity of the NADPH oxidase complex, so they do not significantly inhibit O<sub>2</sub><sup>•−</sup> generation by IC-stimulated neutrophils but probably function as O<sub>2</sub><sup>•−</sup> scavengers. Albeit small, the inhibitory effect of the tested compounds on NADPH oxidase can help modulate ROS production by neutrophils in IC-mediated diseases, since large amounts of these cells infiltrate into the inflammatory site [4,42].

To gain initial insight into the action of 3-phenylcoumarin derivatives in the subsequent steps of ROS generation by IC-stimulated neutrophils, as measured by the CL-lum assay, we evaluated the inhibitory effect of these derivatives on MPO activity and their HOCl scavenging potential. We studied the inhibitory effect of the 3-phenylcoumarin derivatives on MPO activity using a cell-free system and purified MPO, as described in [Experimental procedure](#) section. Inhibition increases with the number of free hydroxyl groups (**19** > **5** > **1**) on the molecule. However, the mono-hydroxylated 3-phenylcoumarin **1** is as effective as the pentahydroxylated flavonoid quercetin. Therefore, not only the number but also the position of the hydroxyl group in a molecule influences its ability to inhibit MPO activity, since compounds **19** and **1** bear two and one *o*-dihydroxyl groups, respectively. These results agree with reports highlighting the importance of the number and position of hydroxyl groups, especially the presence of the catechol group, for efficient inhibition of the catalytic activity of MPO [43] and horseradish peroxidase [12,14]. In addition, the acetylated 3-phenylcoumarin derivatives do not significantly inhibit MPO activity, reinforcing the importance of free hydroxyls for this biological activity.

We also assayed the HOCl scavenging potential of the 3-phenylcoumarin derivatives in a cell-free system. The ranking order of the HOCl scavenging effect suggests that the number and position of free hydroxyl groups are important. The catechol group significantly contributes to the HOCl scavenging effect of flavonols [44] and the free radical scavenging effect of flavonoids [45], coumarins [46], and 3-phenylcoumarins [14,24]. Interestingly, acetylation of the free hydroxyls does not affect the HOCl scavenging potential of the tested 3-phenylcoumarins considerably. Such result agrees with literature reports that *o*-diacetoxy-4-methylcoumarins display strong free radical scavenging activity, which could be compared to the corresponding *o*-dihydroxy parent structures [46,36]. Raj et al. [36] have suggested that the acetyl group spontaneously hydrolyzes in aqueous medium,

generating the active hydroxylated form; however, the exact mechanism underlying the antioxidant effect of acetylated compounds remains unexplained.

Luminol does not specifically detect ROS, so the measured CL-lum results from luminol oxidation by different types of ROS [35]. Considering that MPO and HOCl are the most important mediators of luminol oxidation (and CL-lum production) in activated neutrophils, inhibition of MPO activity and HOCl scavenging might contribute to the inhibitory effect of the 3-phenylcoumarin derivatives on the CL-lum of IC-stimulated neutrophils. Although the acetylated compounds do not inhibit the MPO activity in cell-free systems, intracellular esterases can hydrolyze these compounds, yielding the active hydroxylated product in the neutrophils.

The order of the inhibitory effects of the 3-phenylcoumarin derivatives on the CL-lum and CL-luc of IC-stimulated human neutrophils agrees with previous results on the screening of the modulatory effect of these compounds on rabbit neutrophils [15]. The absolute IC<sub>50</sub> values and the relative influence of acetylation of free hydroxyl groups have little interspecies (humans and rabbits) variation. However, most of the 3-phenylcoumarin derivatives studied here inhibit the human neutrophil CL-lum more than they inhibit the human neutrophil CL-luc, while the opposite trend is detected for rabbit neutrophils. Together, these data suggest that small cellular physiology differences between species [47] can interfere in the structure–activity relationship analysis, although the rabbit is an important experimental model for IC-mediated diseases [48].

The results of the present work suggest that small differences in the structural features of the 3-phenylcoumarin derivatives contribute to inhibition of the CL-luc and CL-lum of IC-stimulated human neutrophils. Considering the set of compounds tested here, compound **5**, bearing the 6,7-dihydroxyl and the 3',4'-methylenedioxy groups, inhibits both CL-lum and CL-luc more effectively, whereas the other compounds inhibit CL-lum more selectively. Their mechanism of action does not involve inhibition of NADPH oxidase activity or cytotoxicity, but we detected significant inhibition of MPO activity and HOCl scavenging ability. This is just a preliminary analysis, so other mechanisms are probably involved in the inhibition of neutrophil ROS generation and merit further investigation.

Considering the important participation of neutrophil-derived ROS in the pathogenesis of IC-mediated diseases and regulation of acute and chronic inflammatory processes, our results can help develop more potent and selective antioxidant and anti-inflammatory drugs based on the 3-phenylcoumarin moiety.

#### 4. Significance

Some inflammatory diseases involve the deposition of immune complexes (IC) in tissues. This event triggers intense neutrophil recruitment and activation, with subsequent release of oxidant and lytic compounds that damage the surrounding tissues. On the other hand, reactive oxygen species (ROS) production by neutrophils is essential for the host defense and regulation of acute and chronic inflammatory responses. Therefore, modulating this neutrophil function is an important therapeutic strategy to manage inflammation and reduce the deleterious effects of ROS. Our research team has been investigating the antioxidant and anti-inflammatory potential of 3-phenylcoumarin derivatives, which have arisen as promising compounds to be used for the aforementioned purpose. The present study reported the modulator effect of ten 3-phenylcoumarin derivatives on the superoxide anion and total ROS generation by IC-stimulated human neutrophils. We found that most compounds did not significantly act at the beginning of neutrophil ROS production process, since they did not significantly inhibit the NADPH oxidase activity. In fact, they affected the latter steps of this process; the 3-phenylcoumarin derivatives that inhibited the overall neutrophil ROS generation process the most strongly also inhibited MPO activity and scavenged HOCl. Interestingly, compound **5**, which

bears 6,7-dihydroxyl and 3',4'-methylenedioxy groups, inhibits the neutrophil ROS generation the most effectively, yielding similar IC<sub>50</sub> values for both steps studied. In addition, the type, number, and position of the hydroxyl and acetoxyl groups influenced the ability of 3-phenylcoumarin derivatives to decrease the ROS generation by IC-stimulated neutrophils, but the structural features contributing to inhibition of the two steps of the process here investigated were a little different. Together, the results of this study contribute to understand the mechanism of action of 3-phenylcoumarin derivatives on neutrophils and also to develop more potent and selective therapeutic molecules that could modulate ROS production by neutrophils in IC-mediated inflammatory diseases.

## 5. Experimental procedure

### 5.1. Chemicals

Diphenyleneiodonium chloride (DPI), guaiacol, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), lucigenin (bis-*N*-methylacridinium nitrate), *N,N*-dimethylformamide (DMF), ovalbumin, quercetin dihydrate (3',4',3,5,7-pentahydroxyflavone), 3,3',5,5'-tetramethylbenzidine (TMB), taurine, Triton X-100, and Trypan Blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), human leukocyte myeloperoxidase (MPO, EC 1.7.1.11), and *p*-aminobenzohydrazide (*p*-AB) were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). The gelatin (microbiological grade) was acquired from Difco Laboratories (Detroit, MN, USA), and LDH Liquiform was provided by Labtest Diagnostica (Lagoa Santa, MG, Brazil). All the other chemicals and solvents used in this work were of analytical grade and purchased from commercial sources.

### 5.2. 3-Phenylcoumarin derivatives

The 3-phenylcoumarin derivatives were synthesized and characterized as previously described [14,32]. The identification code of the compounds is the same as the one employed in a previous work [14] (Fig. 1).

### 5.3. Human neutrophils isolation

Twenty adult subjects in good general health and aged between 18 and 40 years were recruited according to the protocol approved by the local Research Ethics Committee (CEP/FCFRP-USP, protocol number 182). All the participants signed an informed consent prior to the performance of any study-related procedure. Exclusion criteria were as follows: (1) history of any acute or chronic disease, (2) recent use of anti-inflammatory drugs, (3) recent history of alcohol or drug abuse, or (4) active smoking.

Blood was collected from volunteers by venous puncture and placed in Alsever solution (v/v), which served as anticoagulant. Neutrophils were isolated by the method described by Lucisano and Mantovani [49], with modifications [50]. The cell pellets were suspended in Hank's balanced saline solution (HBSS) containing 0.1% (v/v) gelatin (HBSS-gel). The cell preparations contained 80–90% neutrophils with viability higher than 95%, as established by exclusion with Trypan Blue.

### 5.4. Cytotoxicity evaluation

The cytotoxic effect of the 3-phenylcoumarin derivatives on neutrophils was evaluated as described by Lucisano-Valim et al. [51]. Values in parentheses refer to the final concentrations in 0.5 mL of reaction.

Briefly, aliquots of neutrophils ( $1 \times 10^6$  cells/mL) were incubated with a 3-phenylcoumarin derivative (20  $\mu$ M), quercetin (10  $\mu$ M), DMSO (0.1% v/v; vehicle), HBSS-gel (negative control), or Triton X-100 (0.2% v/v; positive control) for 30 min, at 37 °C. The cell pellets

were suspended in HBSS-gel after centrifugation (755  $\times$ g, 10 min, 4 °C), and the cellular viability was determined by the Trypan Blue dye exclusion test, by counting a total of 200 cells for each sample. The activity of cytosolic lactate dehydrogenase (LDH) released into the supernatant was measured on the basis of absorbance changes at 340 nm for 2 min, at 37 °C (U2910 spectrophotometer, Hitachi Instruments, Tokyo, Japan). The LDH Liquiform™ test kit was used in this assay.

### 5.5. Immune complexes preparation

Precipitated immune complexes (IC) of anti-ovalbumin IgG antibody and ovalbumin were prepared from the equivalence zone, as described in a previous work [49]. Total protein concentration in the precipitate was determined by absorbance readings at 280 nm and expressed as  $\mu$ g/mL. The IC were diluted in HBSS pH 7.2 for use.

### 5.6. Neutrophil chemiluminescence assay

The neutrophil chemiluminescence (CL) assay was performed in 96-well microplates using the procedure described by Lucisano-Valim et al. [51], with slight modifications. The concentration of each component in the reaction medium (0.2 mL) is indicated in parentheses below. Lucigenin, luminol, quercetin, and 3-phenylcoumarins stock solutions were prepared in DMSO and diluted in HBSS-gel prior to use.

Aliquots of neutrophils ( $1 \times 10^6$  cells/mL) were mixed with the chemiluminescence (CL) probe [luminol (0.1 mM) or lucigenin (0.1 mM)] and the tested compound — a 3-phenylcoumarin derivative (0.01–20  $\mu$ M), quercetin (0.01–10  $\mu$ M; reference compound), DMSO (0.1% v/v; vehicle), or HBSS-gel (control). The mixture was incubated at 37 °C for 3 min, and the reaction was initiated by adding IC (60  $\mu$ g/mL). The luminol- and lucigenin-enhanced chemiluminescence (CL-lum and CL-luc, respectively) were measured in a microplate luminometer (LB 960 Centro, Berthold Technologies, Bad Wildbad, Germany), and the light emission was recorded in cps (photon counts per second) for 20 min, at 37 °C. The percentage of CL inhibition by each sample was calculated using the formula  $[1 - (\text{AUC}_{\text{sample}}/\text{AUC}_{\text{control}})] \times 100$ . AUC is the area under the time-course curve, and it is used to determine IC<sub>50</sub> values (concentration that inhibits 50% of the neutrophil CL).

### 5.7. NADPH oxidase activity

The NADPH oxidase activity was assessed by measuring O<sub>2</sub> consumption by IC-stimulated neutrophils according to a previously described procedure [50]. Values in parentheses refer to the final concentrations in 0.5 mL. Briefly, the basal O<sub>2</sub> consumption by neutrophils ( $4 \times 10^6$  cells/mL) was measured in the presence of the tested compound — a 3-phenylcoumarin derivative (20  $\mu$ M), quercetin (20  $\mu$ M; reference compound), DPI (1  $\mu$ M; positive control), DMSO (0.1% v/v; vehicle), or HBSS-gel (control) — for 7 min, at 37 °C. The reaction was started by adding IC (240  $\mu$ g/mL) to the neutrophils/tested compound mixture, and O<sub>2</sub> consumption was measured for 7 min, at 37 °C. Oxygen consumption by NADPH oxidase was determined by calculating the difference between the rates before and after IC addition.

### 5.8. Myeloperoxidase activity

The modulator effect of the 3-phenylcoumarin derivatives on human MPO activity was evaluated using the method of Regasini et al. [43], with modifications. Hydrogen peroxide working solutions were prepared daily, and the H<sub>2</sub>O<sub>2</sub> concentration was calculated by measuring its absorbance at 240 nm ( $\epsilon = 43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ) [52].

The MPO activity was assayed in 96-well microplates, and values in parentheses refer to the final concentrations in 0.2 mL. Reaction mixtures containing PBS (10 mM sodium phosphate buffer pH 7.4, 140 mM NaCl, 10 mM KCl), guaiacol (30 mM), MPO (2 nM), and the tested compound

– a 3-phenylcoumarin derivative (20  $\mu\text{M}$ ), quercetin (20  $\mu\text{M}$ ; reference compound), *p*-aminobenzohydrazide (20  $\mu\text{M}$ ; positive control), DMSO (0.1% v/v, vehicle), or PBS (negative control) – were incubated at 25 °C, for 2 min. The reaction was initiated with  $\text{H}_2\text{O}_2$  (500  $\mu\text{M}$ ), and absorbance changes were recorded at 470 nm for 2 min, at 25 °C (Varian Cary 50 Bio UV/Visible spectrophotometer, Sparta, Australia). MPO activity was calculated from the initial linear rate of the reaction.

### 5.9. Hypochlorous acid scavenging assay

The HOCl scavenging potential of the 3-phenylcoumarin derivatives was assessed by the method of Dypbukt et al. [52], with modifications. Domestic sodium hypochlorite, purchased as approximately 350 mM solution in NaOH, was neutralized by dilution in PBS immediately before use. The hypochlorite concentration was determined by measuring its absorbance at 292 nm in a solution at pH 12 ( $\epsilon = 350 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ). Stock solutions of 3-phenylcoumarin derivatives were prepared in DMF and diluted in PBS for use.

The working solution of the tested compound – a 3-phenylcoumarin derivative (0.01–12  $\mu\text{M}$ ), quercetin (0.01–12  $\mu\text{M}$ ), DMF (0.03% v/v, vehicle), or PBS (control) – was added to HOCl (50  $\mu\text{M}$ ) and allowed to react for 10 min. Taurine (5 mM) was then added to the reaction medium, and taurine chloramine was detected after 5 min by rapidly mixing the developing reagent (2 mM TMB in 400 mM acetate buffer pH 5.4 containing 10% DMF and 100  $\mu\text{M}$  KI). The mixture was incubated for 5 min and the absorbance was recorded at 650 nm in a 96-well microplate reader (Varian Cary 50 Bio UV/Visible spectrophotometer, Sparta, Australia). All the reaction steps were conducted in the dark at 25 °C.

### 5.10. Data analysis

Experimental data were processed and analyzed with the aid of the GraphPad Prism Software (version 3.00 for Windows, GraphPad Software Inc., San Diego, CA, USA). Statistical analysis was performed by Student's *t* test (for two groups) or analysis of variance (ANOVA) followed by Tukey's test (for three or more groups), as indicated in the legends.  $p < 0.05$  was considered significant.

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